Detection and immunolabelling of peroxisomal proteins

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Running Head: Localisation of peroxisomal proteins

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Summary

Peroxisomes are essential organelles in mammals which contribute to cellular lipid metabolism and redox homeostasis. The spectrum of their functions in human health and disease is far from being complete, and unexpected and novel roles of peroxisomes are being discovered. To date, those include novel biological roles in anti-viral defence, as intracellular signalling platforms and as protective organelles in sensory cells. Furthermore, peroxisomes are part of a complex network of interacting subcellular compartments which involves metabolic cooperation, cross-talk and membrane contacts. As potentially novel peroxisomal proteins are continuously discovered, there is great interest in the verification of their peroxisomal localisation. Here, we present protocols used successfully in our laboratory for the detection and immunolabelling of peroxisomal proteins in cultured mammalian cells. We present immunofluorescence and fluorescence-based techniques as well as reagents to determine peroxisome-specific targeting and localisation of candidate proteins.

1. Introduction

Peroxisomes are dynamic, multifunctional subcellular organelles that rapidly assemble, multiply and degrade in response to the metabolic needs of the cell. A remarkable feature of peroxisomes is their ability to respond to intra- and extracellular changes and stimuli with alterations in their biogenesis, morphology, number, protein composition and metabolic activity ***(1)***. Peroxisomes contribute to several anabolic and catabolic cellular pathways including the metabolism of fatty acids and reactive oxygen species, which underlines their importance for human health and development ***(2, 3)***. However, the spectrum of peroxisomal tasks is far from being complete. Several unexpected and novel peroxisomal functions have been discovered in recent years, including biological roles in anti-viral defence ***(4)***, as intracellular signalling platforms ***(5-7)*** and as protective organelles in sensory cells, e.g., the hair cells of the inner ear ***(8)***. Those studies and proteomics analyses (***9-11***) have also led to the identification of novel peroxisomal proteins, or the localisation and targeting of known proteins to peroxisomes, which were initially detected in the cytoplasm or associated with other organelles (***12-14***). In this respect, a dual localisation of key components of the organelle division machinery to mitochondria and peroxisomes should be mentioned, which is shared by both organelles (***15***). Peroxisomes are functionally integrated into a complex network of communicating subcellular compartments, and are supposed to interact and cooperate with several other membranous organelles (***16***). Further variability in peroxisomal proteins is detected in individual tissues or organs. The peroxisomal proteome in brain, lung and testis differs significantly from the well-characterized liver and kidney peroxisomes (***17-19***), and novel tissue-specific peroxisomal proteins are continuously identified (***8, 20, 21***).

The recent developments have resulted in a great interest in peroxisomes and their physiological functions as well as in the localisation of potentially novel candidate proteins at peroxisomes. This chapter is intended to provide detailed information on methods and strategies used successfully in our laboratory for the detection and immunolabelling of

peroxisomal proteins in cultured mammalian cells. We present immunofluorescence and fluorescence-based techniques as well as reagents to determine peroxisome-specific targeting and localisation of candidate proteins.

2. Materials

All reagents and equipment used to maintain and process living cells need to be sterile, and appropriate aseptic techniques and practices should be applied at all times.

2.1 Mammalian Cells and Plasmids

1. Mammalian cell line of interest, here: COS-7 (African green monkey kidney cells) (American Type Culture Collection CRL-1651) (*see* **Note 1**).
2. Plasmids for expression of candidate proteins in mammalian cells, here: FLAG-*Hs*ACBD5, Myc-*Hs*ACAD11 (***22***), optional: EGFP- PTS1 (*see* **Notes 2** and **3**).

2.2 Cell Culture Equipment

1. Class II Biological Safety Cabinet/Tissue Culture Hood (*see* **Note 4**).
2. Humidified CO_2 incubator (95% air, 5% CO_2, 37°C).
3. Inverted light microscope (phase contrast).
4. 37°C water bath.
5. Mammalian cell counter.
6. Vacuum aspiration system.
7. Table top centrifuge equipped with a swing-out rotor for 15-ml conical tubes.

2.3 Cell Culture Media, Buffers, and Reagents

1. Complete growth medium: Dulbecco's modified Eagle's medium (DMEM), high glucose (4.5 g/L) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM Glutamine, 100 units/mL penicillin and 100 µg/mL streptomycin (store at 4°C).
2. Phosphate-buffered saline (1×PBS) (without Ca^{2+} and Mg^{2+}): 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 140 mM NaCl, 2.5 mM KCl (store at 4°C).
3. TrypLE™ Express solution (1×) (Gibco) (store at 4°C) (*see* **Note 5**).
4. 70% (v/v) ethanol.
5. Clean and sterile glass coverslips (19 mm ∅, 0.13-0.17 mm thickness) (*see* **Note 6**).
6. Metal tweezers

2.4 Transfection

1. DEAE (Diethylaminoethyl)-Dextran: 50 mg/mL in distilled water (dH_2O). Sterilize by filtration and aliquot. Store at 4ºC.
2. Chloroquin: 60 mg/mL in dH_2O (1000×). Sterilize by filtration and aliquot. Store at -20ºC.
3. Plasmid DNA in sterile, deionized water (1 µg/µL) (*see* **Note 7**).
4. DAPI (diamidino-2-phenylindole): 1 mg/mL in sterile dH_2O (1000×). Store at 4ºC protected from light.

2.5 Fluorescence Microscopy

1. Fluorescence microscope work station, here: inverted IX-81 fluorescence microscope; 150 Watt Xenon-Arc lamp; 10x ocular lens; PlanApo oil immersion objectives with 60× and 100× magnification; equipped with the appropriate sets of excitation filters, dichromatic mirrors and emission filters; CCD digital black and white camera (CoolSNAP HQ^2) driven by Visitron imaging software.
2. Immersion Oil.

3. A humid environment is necessary to prevent evaporation of reagents during incubations. Inexpensive humidity chambers can be generated by putting a moistened filter paper into the lid of a cell culture dish or multi-well plate.
4. 10×PBS (for 1 L): Weigh 80 g of NaCl, 2 g of KCl, 14.4 g of $Na_2HPO_4 \times 2\ H_2O$ and 2 g of KH_2PO_4. Bring volume to 1 L, followed by adjustment of the pH to 7.4. For 1× PBS, dilute 1:10 with dH_2O and adjust pH to 7.4 if necessary. Both solutions are stable at room temperature (RT).
5. PBS-PFA fixative: 4% paraformaldehyde (PFA), 1×PBS. PFA should be handled under a fume hood and glove protection is required. Weigh 4 g of PFA and add to 100 mL of 1×PBS. Add a drop of 1 N NaOH and heat stirring solution at 60°C until PFA dissolves. When solution becomes clear, remove flask from the heating plate, cool to RT, filter through Whatman filter paper and adjust pH to 7.4. This solution is made fresh every time and used at RT.
6. Triton X-100 solution: 0.2% Triton X-100, 1×PBS. Carefully shake until Triton X-100 is completely dissolved. Avoid generating a lot of foam prior to use. This solution is stored at 4°C and used at RT.
7. Digitonin stock solution: 1 mg/mL in dH_2O (400×). Store at -20°C.
8. Blocking solution: 1% Bovine serum albumin (BSA), 1×PBS. Store solution at 4°C or aliquot and freeze. This solution is stable until evidence of bacterial growth.
9. Mowiol Mounting Medium (MMM) for immunofluorescence: To prepare MMM stock, dissolve 12 g of Mowiol 4-88 powder in 40 mL of 1×PBS by stirring overnight at RT. The next day add 20 mL of glycerol and stir overnight. Centrifuge the viscous solution for 1 h at 18,500×*g* (Beckman Avanti Centrifuge J-251, 15,000 rpm with a rotor JA 25.50) to pellet any remaining undissolved Mowiol crystals. Dissolve a few crystals of sodium azide in the supernatant to avoid contamination and aliquot stock. Store at 4°C. To prepare the working solution, mix 3 volumes of MMM stock with 1

volume of n-propyl-gallate stock (anti-fading reagent) and store at 4°C. This solution is stable for 4-6 weeks.

10. 2.5% n-propyl-gallate stock (anti-fading reagent): Dissolve 0.625 g of n-propyl-gallate in 12.5 mL 1×PBS (neutralize pH to dissolve n-propyl-gallate). Add 12.5 mL of glycerol and stir at RT until completely dissolved (*see* **Note 8**). Directly add fresh reagent to MMM working solution. Store the solution protected from light at 4°C. This solution is stable for 4-6 weeks and should be replaced when it turns brownish. Prolonged storage can lead to crystal formation within the solution. Do not try to re-suspend, but avoid to pipet crystals.

2.6 Controls

Negative controls should be performed to assess the level of general background (i. e., incubation with secondary antibodies only). Negative controls using isotype control antibodies or pre-immune serum from the same species as the primary antibody may also be applied to validate the specificity of staining results.

2.7 Antibody sources

1. Myc: mouse monoclonal Myc-epitope antibody (Santa Cruz, sc-40)
2. FLAG: mouse monoclonal FLAG-epitope antibody (Sigma, F3165)
3. Pex14: rabbit polyclonal Peroxin 14 (Pex14) antibody (kindly provided by D. Crane, Brisbane, Australia)
4. Catalase: mouse polyclonal catalase antibody (Abcam, ab88650)
5. ACBD5: rabbit polyclonal Acyl-CoA Binding Domain 5 (ACBD5) antibody (Sigma, HPA012145)
6. ACAD11-B12: mouse monoclonal Acyl-CoA Dehydrogenase 11 (ACAD11) antibody (Santa Cruz, cs-514027).

3. Methods

A convenient method to determine the peroxisomal localization of uncharacterized mammalian candidate proteins is their expression in the form of tagged fusion proteins in mammalian cells and subsequent immunofluorescence microscopy. Colocalisation of the tagged fusion protein with a peroxisomal marker is indicative for peroxisomal localization. As cell-permeable dyes for the *in vivo* staining of mammalian peroxisomes are still not available, the expressed candidate protein and an endogenous peroxisomal marker protein are usually detected by antibody staining.

Alternatively, a fluorescent candidate protein can be co-expressed with a fluorescent peroxisomal marker protein and examined for colocalisation by fixed or live cell imaging. The latter requires specific equipment (inverted microscope, heated microscope stage, temperature and CO_2 control, live cell imaging chamber to insert either cover slips or glass bottom dishes). A multitude of constructs encoding for peroxisome-targeted fluorescent fusion proteins are now commercially available (e.g. EGFP-, DsRed- or mCherry-tagged peroxisomal fusion proteins). EGFP (and its derivatives) bearing a peroxisomal targeting signal 1 (PTS1) composed of amino acids SKL at the very C-terminus (EGFP-PTS1) is commonly used to label the peroxisomal matrix.

It is recommended to confirm the peroxisomal localization of the expressed candidate protein by detection of the endogenous protein. When suitable antibodies directed against the candidate protein of interest are available, the endogenous protein can be colocalised with a peroxisomal marker protein using immunofluorescence microscopy. It should be considered that the candidate protein may be expressed in a cell type-, tissue- or species-specific manner.

3.1 Cell Culture

1. Perform all cell culture related work in a Class II Biological Safety Cabinet/Tissue Culture Hood and disinfect the work surface and materials (e.g., pipettes) with 70% (v/v) ethanol.
2. Grow COS-7 cells in complete growth medium (10 cm ∅ cell culture dishes) (*see* **Note 9**) in a humidified CO_2 incubator (95% air, 5% CO_2, 37°C).
3. Refresh the cell culture medium every 2-3 days.
4. Split the cells before they reach 100% confluency (*see* **steps 4** to **14**).
5. Pre-warm 1×PBS, TrypLE™ Express solution, and complete growth medium to 37°C.
6. Remove all medium from the cell culture dish with a Pasteur pipette by vacuum aspiration and wash the cells once with 5 mL of 1×PBS.
7. Add TryplExpress solution to the cells and gently tilt to cover the surface (1 mL/10 cm dish).
8. Incubate the cells for 2-5 min at 37°C (*see* **Note 10**).
9. Upon detachment, harvest the cells in complete growth medium (10 mL/10 cm dish) (*see* **Note 11**).
10. Carefully resuspend the cells by pipetting the cell suspension 2-3 times up and down and further detach remaining cells from the surface of the dish. Repeat in case the cells still form aggregates or clumps.
11. Transfer the cells to a 15-mL conical tube and take an aliquot of the suspension for cell counting.
12. Pellet the cells by centrifugation (500×*g*, 3 min at RT).
13. Resuspend the cell pellet in 10 mL of complete medium.
14. For maintenance, transfer the required amount of cells (approx. 3-5 × 10^5 cells) to a new 10 cm cell culture dish containing 10 mL of complete medium (*see* **step 4**) (*see* **Note 12**).

15. For transfection with plasmid DNA and/or immunofluorescence microscopy, seed the required amount of cells (2.5×10^5 cells per 6 cm dish) on sterile, clean glass coverslips in 6 cm ∅ cell culture dishes (*see* **Note 13**). We routinely use round coverslips (19 mm ∅, 0.13-0.17 mm thickness) and add 3-4 coverslips per 6 cm dish prior to seeding of the cells using sterile tweezers (*see* **Notes 14** and **15**).
16. Incubate the cells in a humidified CO_2 incubator (95% air, 5% CO_2, 37°C) for 24 h to allow attachment to the coverslips.

3.2 Transfection of COS-7 cells with DEAE-Dextran (*see* **Notes 16** and **17**)

1. Perform all transfection related work in a Class II Biological Safety Cabinet/Tissue Culture Hood (see **3.1, step 1** and **Note 4**).
2. For one 6 cm dish, mix 4 µg of plasmid DNA with 0.5 mL DMEM (without FBS and antibiotics) in a sterile 1.5 mL conical microcentrifuge tube by carefully pipetting up and down 2-3 times (*see* **Note 18**).
3. Add 3 µL DEAE-Dextran. Invert tube 3 times and incubate for 15 min at RT (*see* **Note 18**).
4. Shortly before the end of the incubation time remove all medium from the cell culture dish with a Pasteur pipette by vacuum aspiration and wash the cells twice with 2 mL of 1×PBS.
5. Add the transfection mixture dropwise to the cells. Avoid pipetting up and down, as this will destroy the DNA-DEAE-Dextran complexes.
6. Incubate the cells in a humidified CO_2 incubator (95% air, 5% CO_2, 37°C) for 1.5 h. Carefully tilt the dish every 15 min and ensure that the cells are properly covered by fluid (*see* **Note 19**).
7. Add the end of the incubation time, add 4 mL complete growth medium containing 4 µL chloroquine stock solution (*see* **Note 20**).

8. Incubate the cells in a humidified CO_2 incubator (95% air, 5% CO_2, 37°C) for 2.5-3 h.
9. Remove all medium from the cell culture dish by vacuum aspiration and wash the cells once with 2 mL of 1×PBS.
10. Add 4 mL of complete growth medium and return cells to the CO_2 incubator. Incubate for 24-48 h (*see* **Note 21**).

3.2.1 Evaluation of efficient transfection and expression of fluorescent candidate fusion proteins (optional) (*see* **Note 22**)

1. In the Tissue Culture Hood, transfer a coverslip from a 6 cm dish containing transfected cells to a new dish (or multi-well plate) containing 1×PBS. The following steps can be performed under non-sterile conditions.
2. Remove the 1×PBS from the dish with a Pasteur pipette by vacuum aspiration and immediately fix cells with 4% PFA for 20 min at RT (*see* **protocol 3.3, step 2**).
3. Wash sample 3 times with 1×PBS for 2-5 min.
4. Incubate the sample for 1-3 min in DAPI working solution (dilute DAPI stock solution 1:1000 in 1×PBS) (*see* **Note 23**).
5. Aspirate the DAPI solution from the dish and wash the cells briefly 2-3 times with 1×PBS.
6. Rinse the coverslip briefly with dH_2O and mount on glass slides for microscopic analysis (*see* **protocol 3.3**, **steps 13-16**) (*see* **Note 24**)
7. Choose the appropriate microscope settings to image both the DNA-DAPI complexes (all cellular nuclei) and the expressed fluorescent candidate fusion protein of interest. The excitation/emission wavelengths for DAPI are 360/460 nm.
8. Adjust the exposure times according to the brightness of the fluorescence signals and collect multicolor images.

9. Determine the transfection efficiency by calculating the fraction of double-labeled cells (count at least 50 randomly selected cells) (*see* **Note 25**).

3.3 Detection of expressed (or endogenous) peroxisomal membrane proteins (*see* **Note 26**)

1. Seed COS-7 cells on coverslips (*see* **protocol 3.1**) and after 24 h transfect COS-7 cells with a mammalian expression vector encoding a tagged version of your candidate peroxisomal membrane protein of interest (here, Flag-ACBD5) (*see* **protocol 3.2**). When designing the expression construct, ensure that the tag does not interfere with membrane insertion of the fusion protein. Expression of both N- and C-terminally tagged fusion proteins may be required. Besides wild type proteins, mutated versions can as well be used to gather information on targeting signals. Expression of some peroxisomal membrane proteins can result in organelle clustering and aggregation (see **Fig. 1A**).
2. All following steps can be performed under non-sterile conditions. Remove the culture medium from the 6 cm dish and briefly wash the cells (on glass coverslips) with 1×PBS to remove serum proteins. Take care that the cells never get dry during the experimental procedure.
3. Fix the cells immediately with fresh 4% paraformaldehyde (PFA) for 20 min at RT (*see* **Note 27**).
4. Wash samples 3 times in 1×PBS for 2-5 min.
5. Permeabilize the cells with 0.2% Triton X-100 in 1×PBS for 10 min at RT (*see* **Note 28**).
6. Wash samples 3 times in 1×PBS for 2-5 min.
7. Block in 1% BSA in 1×PBS for 10 min at RT (*see* **Note 29**).
8. Wash samples 3 times in 1×PBS for 2-5 min.
9. Using tweezers, place the cover slips in a humid chamber (cell-side up!). Apply minimal forces, as the cover slips will break very easily (*see* **Note 30**).

10. Apply antibodies diluted in 1% BSA/1×PBS (here, rabbit polyclonal anti-Pex14, 1:1400; mouse monoclonal anti-Flag, 1:500). Apply carefully without making air bubbles and ensure that the cells on the cover slip are completely covered by the antibody solution. In most instances, we apply primary antibodies for 1 h at RT. Antibody dilutions can be prepared before the experiment or during the fixation period. Keep the antibodies on ice prior to use (*see* **Notes 31-32**).
11. Wash samples 3 times in 1×PBS for 2-5 min.
12. Incubate cells with fluorophore-conjugated secondary antibodies diluted in 1×PBS for 1 h at RT (e.g., Donkey-anti-Rabbit-Alexa594, Donkey-anti-Mouse-Alexa488). During the incubation, the humid chamber should be covered (e.g. with aluminium foil or a box) to avoid exposure to room light and potential photobleaching of the fluorophores. Keep the antibody dilutions on ice and in the dark prior to use.
13. Wash samples 3 times in 1×PBS for 2-5 min (*see* **Note 33**).
14. Apply a drop of Mowiol 4-88 on a glass microscope slide. Glass slides should be clean and dust-free. Label the slides with a waterproof marker or pencil (*see* **Note 34**).
15. Using tweezers dip the cover slip briefly into dH_2O and remove excess water by holding a piece of filter paper close to the rim of the cover slip.
16. Quickly place the cover slip (cell-side down!) onto the drop of Mowiol 4-88 on the glass slide. Avoid enclosing air bubbles underneath. Using the tips of the tweezers, minimal pressure can be applied to the top of the cover slip to remove air bubbles. Remove excess Mowiol 4-88 (*see* **Note 35**).
17. Put slides in a lightproof slide box and let Mowiol 4-88 dry for 2-3 h. Store slides at 4°C in the dark until analysis (*see* **Note 36**).
18. Microscopic analysis: choose the appropriate microscope settings to image both the peroxisomal marker protein and the exogenously expressed (or endogenous) candidate protein of interest.

19. Adjust the exposure times according to the brightness of the fluorescence signals and collect multicolor images (**Fig. 1A, B**).
20. The transfection efficiency can be determined by calculating the fraction of double-labelled cells (count at least 50 randomly selected cells) (*see* **Note 25**).

3.3.1 Topology analysis of tagged peroxisomal membrane proteins by differential permeabilisation

1. Seed COS-7 cells on coverslips (*see* **protocol 3.1**) and after 24 h transfect COS-7 cells with a mammalian expression vector encoding a) an N-terminally Myc-tagged version of your candidate peroxisomal membrane protein of interest, b) a C-terminally Myc-tagged version of your candidate peroxisomal membrane protein of interest (*see* **protocol 3.2**). Untransfected or mock transfected COS-7 cells serve as controls. For the following, two sets of cells (each set consisting of control cells, expression of N-terminally Myc-tagged version, expression of C-terminally Myc-tagged version) for differential permeabilisation are required (*see* **Note 37**).
2. Wash both sets of cells with 1×PBS, fix with 4% PFA (for 20 min at RT) and wash samples 3 times in 1×PBS for 2-5 min (*see* **protocol 3.3**, **steps 1-3**).
3. Permeabilize set 1 of cells with 0.2% Triton X-100 in 1×PBS for 10 min at RT.
4. Permeabilize set 2 of cells with 2.5 µg/mL Digitonin in 1×PBS for 5 min at RT (*see* **Note 38**).
5. Wash samples in 1×PBS and block in 1% BSA as described (*see* **protocol 3.3, steps 5-8**).
6. Apply antibodies diluted in 1% BSA/1×PBS. Incubate the control coverslips from each set with a mixture of rabbit polyclonal anti-Pex14, 1:1400 and mouse polyclonal anti-catalase, 1:150. Incubate the Myc-expressing cells (N- and C-tagged versions) from both sets with a mixture of rabbit polyclonal anti-Pex14, 1:1400 and mouse monoclonal anti-

Myc, 1:200. Incubate with primary antibodies for 1 h at RT (*see* **protocol 3.3, step 9**) (*see* **Note 39**).

7. Wash samples 3 times in 1×PBS for 2-5 min.
8. Incubate cells with fluorophore-conjugated secondary antibodies diluted in 1×PBS for 1 h at RT. For controls for example, Donkey-anti-Rabbit-Alexa488, Donkey-anti-Sheep-TRITC; for Myc-expressing cells Donkey-anti-Rabbit-Alexa488, Donkey-anti-Mouse-Alexa594 (*see* **protocol 3.3, step 11**).
9. Wash in 1×PBS, rinse in dH_2O and mount coverslips on microscope slides using Mowiol 4-88 (*see* **protocol 3.3, steps 12-16**).
10. Microscopic analysis: The N- and C-terminal Myc-tag is accessible to anti-Myc antibodies after permeabilization with Triton X-100. Accessibility of the N- and C-terminal Myc-tag after permeabilization with digitonin indicates that both the N- and the C-terminus of the candidate protein are exposed to the cytosol. Inaccessibility of either the N- or the C-terminal Myc-tag indicates exposure to the peroxisomal matrix (or inaccessibility within the peroxisomal membrane). The peroxisomal matrix enzyme catalase, which is used as a control, should only be accessible to anti-catalase antibodies after permeabilization of the peroxisomal membrane with Triton X-100 but not after treatment with digitonin. The peroxisomal membrane protein Pex14, which is used as a peroxisomal membrane marker, should label peroxisomes under both permeabilization conditions (*see* **Note 40**).

3.4 Detection of expressed (or endogenous) peroxisomal matrix proteins

1. Seed COS-7 cells on coverslips (*see* **protocol 3.1**) and after 24 h transfect COS-7 cells with a mammalian expression vector encoding an N-terminally tagged version of your candidate peroxisomal matrix protein of interest (here, Myc-ACAD11) (*see* **protocol 3.2**). As the vast majority of the mammalian peroxisomal matrix proteins contain a peroxisomal

targeting signal (PTS1) at the very C-terminus, tagging at the C-terminal domain (for example with GFP) interferes with peroxisomal targeting via the peroxisomal import receptor Pex5. However, C-terminally tagged versions can be used as negative controls (***22***). It is also possible that candidate proteins are dually targeted to peroxisomes (via a C-terminal PTS1) and mitochondria (via an N-terminal mitochondrial targeting signal). In cases were both peroxisomal and mitochondrial targeting information is predicted (using PTS1 predictor algorithms and Mitoprot2 or Predotar1.03), N- and C-terminally tagged versions can be expressed: N-terminal tagging should permit peroxisomal targeting (but inhibit mitochondrial localisation), whereas C-terminal tagging should permit mitochondrial targeting (but inhibit peroxisomal localisation).

2. Wash cells with 1×PBS and place them in 4% PFA for 20 min at RT (*see* **Note 41**).
3. Wash samples 3 times in 1×PBS for 2-5 min.
4. Permeabilize cells with 0.2% Triton X-100 in 1×PBS for 10 min at RT (*see* **Note 28**).
5. Wash samples 3 times in 1×PBS for 2-5 min.
6. Block in 1% BSA in 1×PBS for 10 min at RT (*see* **Note 29**).
7. Wash samples 3 times in 1×PBS for 2-5 min.
8. Place the coverslips in a humid chamber (cell-side up!) (*see* **protocol 3.3, step 8**) (*see* **Note 30**).
9. Apply antibodies diluted in 1% BSA/1×PBS (here, rabbit polyclonal anti-Pex14, 1:1400; mouse monoclonal anti-Myc, 1:200) and incubate for 1 h at RT (*see* **protocol 3.3, step 9**) (*see* **Notes 31-32**).
10. Wash samples 3 times in 1×PBS for 2-5 min.
11. Incubate cells with fluorophore-conjugated secondary antibodies (e.g., Donkey-anti-Rabbit-Alexa594, Donkey-anti-mouse-Alexa488) diluted in 1×PBS for 1 h at RT.
12. Wash samples 3 times in 1×PBS for 2-5 min (*see* **Note 33**)
13. Rinse cover slips with dH_2O.

14. Mount coverslips (cell-side down!) on a glass slides using Mowiol 4-88 (*see* **protocol 3.3, steps 12-16**).
15. Microscopic analysis: choose the appropriate microscope settings to image both the peroxisomal marker protein and the exogenously expressed (or endogenous) candidate matrix protein of interest (*see* **Note 42**).
16. Adjust the exposure times according to the brightness of the fluorescence signals and collect multicolor images (**Fig. 1C, D**).
17. The transfection efficiency can be determined by calculating the fraction of double-labelled cells (count at least 50 randomly selected cells) (*see* **Note 25**).

4. Notes

1. If possible, a permanent, adherent cell line should be selected which is easy to transfect with plasmid DNA and contains an elaborate peroxisomal compartment. As peroxisomes are very prominent in liver and kidney, permanent hepatocyte- or kidney-derived cell lines are suitable cell models. We have successfully used HepG2 cells (human hepatoblastoma cells, ATCC HB-8065) for our studies (***23***). They can be transfected by PEI (***13***), lipofection or electroporation (***24***). Primary cells such as patient skin fibroblast are difficult to transfect, but high transfection rates have been achieved by microporation (***25***, and this issue).
2. The plasmid encoding peroxisomal EGFP was designed by adding SKL, the prototypic C-terminal targeting signal for peroxisomal matrix proteins, to the C-terminus of Green Fluorescent Protein (***26***).
3. Plasmids designed for mammalian expression of fluorescent peroxisomal marker proteins, which target the peroxisomal matrix, or for the generation of tagged fluorescent or non-fluorescent fusion proteins are commercially available.

4. Follow the biosafety and GMO guidelines of your institution.
5. TrypLE™ Express (12604013, Gibco) is an animal origin-free, RT-stable, recombinant enzyme suitable for the dissociation of a wide range of adherent mammalian cells. It cleaves peptide bonds on the C-terminal sides of lysine and arginine, and is a direct replacement for trypsin. Its high purity increases specificity and reduces damage to cells that can be caused by other enzymes present in some trypsin extracts. Alternatively, Trypsin/EDTA solution (1x) can be used: 0.05% (w/v) trypsin, 0.68 mM EDTA, 5.5 mM glucose, 137.93 mM NaCl, 5.36 mM KCl, 6.9 mM $NaHCO_3$ (store at -20°C).
6. For sterilisation, glass coverslips are put in a glass petri dish, wrapped in tin foil and dry-sterilised for 6 h at 180°C.
7. Prepare transfection-quality plasmid DNA with your method of choice. Plasmid DNA is diluted in sterile, deionised water (recommended for transfection by electroporation or microporation).
8. We usually stir for 2-3 h at RT, but stirring overnight is sometimes recommended.
9. Alternatively, 5 ml/T-25 or 15 ml/T-75cell culture flasks can be used.
10. Carefully check on an inverted microscope if and when the cells start to detach. You can gently tap against the side of the dish to improve detachment; however, tapping may stress sensible cells. It is recommended to further detach cells by gently pipetting complete medium against the surface of the dish while resuspending the cells. Note that extended trypsinization decreases cell viability; the cells should only be exposed to the trypsinization solution until they start to detach.
11. FBS contains trypsin inhibitors, which inhibit trypsin activity.
12. Regularly check that the cells are not contaminated with bacteria (culture medium quickly turns yellow (acidic) and cloudy!), yeast, fungi (microscopic evaluation) or mycoplasms. It is recommended to perform a mycoplasm test on a regular basis. Tests based on DNA-staining (e.g., with DAPI or Hoechst H33258) can be inconclusive; PCR-based tests are

more reliable. Always ensure that the cultured cells are healthy and actively growing (recommended confluency: 70-80%), as all the subsequent steps strongly depend on healthy cells and good cell culture practice.

13. Some cell lines do not attach or spread well on glass coverslips. To improve attachment and cell growth, coverslips can be coated with poly-L-lysine, collagen or an extract of extracellular matrix proteins (e.g., matrigel) (***27***).
14. Depending on the application, glass coverslips of different sizes (e.g., smaller to fit into multi-well plates) and forms (e.g. square ones) can be used. Smaller coverslips require less antibody solution. We prefer slightly bigger coverslips, which harbour more cells, and are thus well suited for morphological quantification of cellular (organelle) parameters.
15. Ensure that the glass coverslips do not float or overlap. The latter will result in areas without attached cells. Adding the coverslips before the medium helps to keep them at the bottom of the dish. Use sterile tweezers to adjust floating or overlapping coverslips.
16. Transfection with DEAE-Dextran is a very simple, reproducible, and low cost method, which works very well with COS-7 cells. Disadvantages may include cytotoxicity and low transfection efficiency for a range of other cell types. DEAE-Dextran is a polycationic derivative of the carbohydrate polymer dextran. The cationic molecule forms complexes with the negatively charged plasmid DNA. The net positive charge of the DNA-DEAE-Dextran complexes allows binding to the cell membrane, where the complexes enter the cell via endocytosis and the DNA is released into the cytoplasm.
17. Alternative transfection methods are PEI transfection, an easy and low-cost method, calcium-phosphate precipitation, lipofection or electroporation. The latter method can result in high transfection rates and usually requires non-adherent cells (e.g. suspension cultures or trypsinization of adherent cells) (***28***). Electroporation of mammalian cells typically requires optimization of various parameters that affect DNA uptake and cell

viability (e.g. electroporation medium, cell type and number, voltage, capacitance, resistance). It is advisable to test and optimize transfection efficiency.

18. The mixture can be scaled up. For example, a mix of 10 µg of plasmid DNA, 1.5 mL DMEM (without FBS and antibiotics) and 9 µL DEAE-Dextran is sufficient for the transfection of three 6 cm dishes (0.5 mL transfection mix per 6 cm dish) or one 10 cm dish (cells numbers as indicated in **3.1**).
19. As the DNA-DEAE-Dextran complexes are entering the cell via endocytosis, it is important to allow interaction with the cell membrane and proper distribution of the complexes.
20. Chloroquine is a lysosomal inhibitor which prevents endosomal acidification. This results in the inhibition of lysosomal enzymes that require an acidic pH, and prevents fusion of endosomes and lysosomes. Addition of chloroquine during the transfection procedure prevents lysosomal degradation of the DNA-DEAE-Dextran complexes.
21. Incubation times depend on the expression level and efficiency of peroxisomal targeting of the expressed protein of interest.
22. Subsequent microscopic localisation studies do not necessarily require very high transfection efficiencies. However, it is recommended to confirm successful transfection and protein expresssion prior to immunofluorescence microscopy, in particular when new DNA constructs are used.
23. DAPI is a cell-permeable blue fluorescent nucleic acid stain that allows visualization of nuclei in fixed cells.
24. Ensure that the mounting medium has dried and is no longer fluid before you add immersion oil and start microscopic analysis.
25. In general, transfection efficiencies between 70-90% are obtained.
26. A related protocol has already been published by the corresponding author in a former issue of this journal (***28***). However, to provide the reader with all relevant practical

information within the same Chapter, the procedure is outlined again here with specific modifications.

27. Fixation with alcoholic fixatives only (e.g., ethanol or methanol) is not recommended, as it interferes with peroxisome morphology (***29***). Some primary antibodies, however, do not work after PFA fixation. In those cases, a combined PFA-methanol fixation can be performed. Cells are first fixed with 4% PFA as indicated, washed 3 times with 1×PBS, and are afterwards post-fixed (and permeabilized) with absolute methanol (-20°C) for 5 minutes. A further permeabilization step can be omitted. In addition, denaturing methods to expose hidden antigens in PFA-fixed cultured cells can be applied (***30***).
28. Instead of Triton X-100, cells can be permeabilized with 2.5 µg/mL digitonin in 1×PBS for 5 min at RT. This is recommended for the localization of mammalian Pex11pβ, which is extracted from peroxisomal membranes of fixed cells during Triton X-100 permeabilization (***31***). For the detection of matrix proteins, permeabilization with Triton X-100 is recommended.
29. Instead of 1% BSA, blocking of free aldehyde groups can be performed with 1% glycine in 1×PBS.
30. Cells grown on coverslips can be incubated with antibodies cell-side up in uni- or multi-well plastic dishes. Alternatively, coverslips can be placed cell-side down on a drop of antibody solution applied on parafilm.
31. Antibody dilutions should be optimized using recommended concentrations as a guideline. A dilution series of all primary and secondary antibodies can be performed.
32. Primary antibody incubations can also be performed at 4°C overnight in a refrigerator or a cold room. In urgent cases, antibody incubations can be performed at 37°C for 30 min in an incubator. In both cases evaporation of the antibody solution should be minimized (e.g. by covering with parafilm or by increasing the volume of the antibody solution applied).

33. At this step, cellular nuclei can be stained by incubating the cells with DAPI or Hoechst H33258.
34. If necessary, glass microscope slides can be cleaned with 70% ethanol, dried and cleaned with (lense) paper.
35. Excess Mowiol 4-88 can be removed either by aspiration with a vacuum pump using a pasteur pipette or plastic pipetting tip or, less elegant, by using a piece of filter paper. Do not leave mounting medium on top of the coverslip as it gets dry and interferes with microscopic observation.
36. Proper mounting in Mowiol 4-88 (containing fresh n-propyl-gallate as antifading reagent) and proper storage at 4°C in the dark can protect the samples for several months or even years.
37. It is recommended to use small tags such as Myc or FLAG. However, larger, fluorescent tags such as GFP can be detected by anti-GFP-specific antibodies.
38. In contrast to Triton X-100, digitonin does not permeabilize the peroxisomal membrane (differential permeabilization). Only epitopes accessible on the cytosolic surface of the peroxisomal membranes will be detected (***24***).
39. Ensure that the antibody for detection of the peroxisomal membrane marker protein (here: anti-Pex14) is directed against an epitope which is accessible on the cytosolic surface of the peroxisomal membrane.
40. It is recommended to confirm the membrane topology by biochemical (for example, protease protection assay) (*see* this issue) and bioinformatics analyses.
41. To improve visualization of the peroxisomal pool of proteins, cytosolic background (e.g., due to accumulation of peroxisomal proteins in the cytoplasm) can be reduced by treatment of cells with digitonin or removal of cytosol by streptolysin O in a semi-permeable system (***32, 33***).

42. Elongated peroxisomal membrane domains can be devoid of peroxisomal matrix proteins under certain conditions. Furthermore, catalase expression may vary depending on the cell type and cell lines selected.

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References

1. Schrader, M., Costello, J., Godinho, L. F., Azadi, A. S, and Islinger, M. (2016) Proliferation and fission of peroxisomes – an update. *Biochim. Biophys. Acta – Molecular Cell Research 1863*, 971-983.
2. Wanders, R. J. A., and Waterham, H. R. (2006) Biochemistry of mammalian peroxisomes revisited. *Annu. Rev. Biochem. 75*, 295-332.
3. Islinger, M., and Schrader, M. (2011): Quick Guide Peroxisomes. *Current Biology 21 (19)*, R800-R801.
4. Dixit, E., Boulant, S., Zhang, Y., Lee, A. S., Odendall, C., Shum, B., Hacohen, N., Chen, Z. J., Whelan, S. P., Fransen, M., Nibert, M. L., Superti-Furga, G., and Kagan, J. C. (2010) Peroxisomes are signaling platforms for antiviral innate immunity. *Cell 141*, 668-681.
5. Diano S., Liu, Z. W., Jeong, J. K., Dietrich, M. O., Ruan, H. B., Kim, E., Suyama, S., Kelly, K., Gyengesi, E., Arbiser, J. L., Belsham, D. D., Sarruf, D. A., Schwartz, M. W., Bennett, A. M., Shanabrough, M., Mobbs, C. V., Yang, X., Gao, X. B., and Horvath, T. L. (2011) Peroxisome proliferation-associated control of reactive oxygen species sets melanocortin tone and feeding in diet-induced obesity. *Nat. Med. 17(9)*, 1121-7.
6. Fransen, M., Nordgren, M., Wang, B., and Apanasets, O. (2012) Role of peroxisomes in ROS/RNS-metabolism: implications for human disease. *Biochim. Biophys. Acta 1822(9)*, 1363-73.
7. Mast, F. D., Rachubinski, R. A., and Aitchison, J. D. (2015) Signaling dynamics and peroxisomes. *Curr. Opin. Cell Biol. 35*, 131-6.
8. Delmaghani, S., Defourny, J., Aghaie, A., Beurg, M., Dulon, D., Thelen, N., Perfettini, I., Zelles, T., Aller, M., Meyer, A., Emptoz, A., Giraudet, F., Leibovici, M., Dartevelle, S., Soubigou, G., Thiry, M., Vizi, E. S., Safieddine, S., Hardelin, J. P., Avan, P., and Petit, C. (2015) Hypervulnerability to Sound Exposure through Impaired Adaptive Proliferation of Peroxisomes. *Cell 163(4)*, 894-906.
9. Gronemeyer, T., Wiese, S., Ofman, R., Bunse, C., Pawlas, M., Hayen, H., Eisenacher, M., Stephan, C., Meyer, H. E., Waterham, H. R., Erdmann, R., Wanders, R. J., and Warscheid, B. (2013) The proteome of human liver peroxisomes: identification of five new peroxisomal constituents by a label-free quantitative proteomics survey. *PLoS One 8(2)*, e57395.
10. Wiese, S., Gronemeyer, T., Ofman, R., Kunze, M., Grou, C. P., Almeida, J. A., Eisenacher, M., Stephan, C., Hayen, H., Schollenberger, L., Korosec, T., Waterham, H. R., Schliebs, W., Erdmann, R., Berger, J., Meyer, H. E., Just, W., Azevedo, J. E., Wanders, R. J., and Warscheid, B. (2007) Proteomics characterization of mouse kidney peroxisomes by tandem mass spectrometry and protein correlation profiling. *Mol. Cell. Proteomics 6(12)*, 2045-57.
11. Islinger, M., Lüers, G. H., Li, K. W., Loos, M., and Völkl, A. (2007) Rat liver peroxisomes after fibrate treatment. A survey using quantitative mass spectrometry. *J. Biol. Chem. 282(32)*, 23055-69.
12. Islinger, M., Lüers, G. H., Zischka, H., Ueffing, M., and Völkl, A. (2006) Insights into the membrane proteome of rat liver peroxisomes: microsomal glutathione-S-transferase is shared by both subcellular compartments. *Proteomics 6(3)*, 804-16.
13. Koch, A., Thiemann, M., Grabenbauer, M., Yoon, Y., McNiven, M. A., and Schrader, M. (2003) Dynamin-like protein 1 is involved in peroxisomal fission. *J. Biol. Chem. 278*, 8597-605.

14. Koch, A., Yoon, Y., Bonekamp, N. A., McNiven, M. A., and Schrader, M. (2005) A role for Fis1 in both mitochondrial and peroxisomal fission in mammalian cells. *Mol. Biol. Cell 16(11)*, 5077-86.
15. Schrader, M., Costello, J., Godinho, L. F., and Islinger, M. (2015) Peroxisome-Mitochondria Interplay and Disease. *J. Inherit. Metab. Dis. 38(4)*, 681-702.
16. Schrader, M., Godinho, L. F., Costello, J., and Islinger, M. (2015) The different facets of organelle interplay—an overview of organelle interactions. *Front. Cell Dev. Biol. 3*, 56.
17. Islinger, M., Cardoso, M. J., and Schrader, M. (2010) Be different—the diversity of peroxisomes in the animal kingdom. *Biochim Biophys Acta 1803*, 881–897.
18. Karnati, S., and Baumgart-Vogt, E. (2009) Peroxisomes in airway epithelia and future prospects of these organelles for pulmonary cell biology. *Histochem. Cell Biol. 131*, 447–454.
19. Dastig, S., Nenicu, A., Otte, D. M., Zimmer, A., Seitz, J., Baumgart-Vogt, E., and Luers, G. H. (2011) Germ cells of male mice express genes for peroxisomal metabolic pathways implicated in the regulation of spermatogenesis and the protection against oxidative stress. *Histochem. Cell Biol. 136*, 413–425.
20. Grzmil, P., Burfeind, C., Preuss, T., Dixkens, C., Wolf, S., Engel, W., and Burfeind, P. (2007) The putative peroxisomal gene Pxt1 is exclusively expressed in the testis. *Cytogenet. Genome Res. 119*, 74–82.
21. Kaczmarek, K., Niedzialkowska, E., Studencka, M., Schulz, Y., and Grzmil, P. (2009) Ccdc33, a predominantly testis-expressed gene, encodes a putative peroxisomal protein. *Cytogenet. Genome Res. 126*, 243–252.
22. Camões, F., Islinger, M., Guimarães, S. C., Kilaru, S., Schuster, M., Godinho, L., Steinberg, G., and Schrader, M. (2015) New insights into the peroxisomal protein inventory: Acyl-CoA oxidases and –dehydrogenases are an ancient feature of peroxisomes. *Biochim. Biophys. Acta 1853(1)*:111-25.
23. Schrader, M., Baumgart, E., Völkl, A., and Fahimi, H. D. (1994) Heterogeneity of peroxisomes in human hepatoblastoma cell line HepG2. Evidence of distinct subpopulations. *Eur. J. Cell Biol. 64*, 281-294.
24. Schrader, M., Reuber, B. E., Morrell, J. C., Sanchez, G.-G., Obie, C., Stroh, T. A., Valle, D., Schroer, T. A., and Gould, S. J. (1998) Expression of PEX11ß mediates peroxisome proliferation in the absence of extracellular stimuli. *J. Biol. Chem. 273*, 29607-29614.
25. Ivashchenko, O., Van Veldhoven, P. P., Brees, C., Ho, Y. S., Terlecky, S. R., and Fransen, M. (2011) Intraperoxisomal redox balance in mammalian cells: oxidative stress and interorganellar cross-talk. *Mol. Biol. Cell 22*, 1440–1451.
26. Koch, A., Schneider, G., Luers, G. H., and Schrader, M. (2004) Peroxisome elongation and constriction but not fission can occur independently of dynamin-like protein 1. *J. Cell Sci. 117*, 3995-4006.
27. Aroso, M., Agricola, B., Hacker, C., and Schrader, M. (2015) Proteoglycans support proper granule formation in pancreatic AR42J cells. *Histochem. Cell Biol. 144(4)*, 331-46.
28. Bonekamp, N., Islinger, M., Gomez-Lazaro, M., and Schrader, M. (2013) Cytochemical detection of peroxisomes and mitochondria. *Methods Mol. Biol. 931*, 467-82.
29. Schrader, M., Baumgart, E., and Fahimi, H. D. (1995) Effects of fixation on the preservation of peroxisomal structures for immunofluorescence studies using HepG2 cells as a model system. *Histochem. J. 27*, 615-619.

30. Peranen, J., Rikkonen, M., and Kaariainen, L. (1993) A method for exposing hidden antigenic sites in paraformaldehyde-fixed cultured cells, applied to initially unreactive antibodies, *J. Histochem. Cytochem. 41*, 447-454.
31. Schrader, M., Almeida, M., and Grille, S. (2012) Postfixation detergent treatment liberates the membrane modelling protein Pex11β from peroxisomal membranes. *Histochem. Cell Biol. 138*, 541-547.
32. Dodt, G., and Gould, S. J. (1996) Multiple PEX genes are required for proper subcellular distribution and stability of Pex5p, the PTS1 receptor: evidence that PTS1 protein import is mediated by a cycling receptor. *J. Cell Biol. 135*, 1763-74.
33. Wendland, M., and Subramani, S. (1993) Cytosol-dependent peroxisomal protein import in a permeabilized cell system. *J. Cell Biol. 120(3)*, 675-85.

Figure Legend

Figure 1. Localization of expressed and endogenous candidate peroxisomal membrane and matrix proteins in mammalian cells.

(A, B) The tail-anchored membrane protein Acyl-CoA Binding Domain 5 (ACBD5) localizes to peroxisomes. **(A)** Peroxisomal localisation of expressed FLAG-ACBD5 in COS-7 cells. Cells were transfected with FLAG-ACBD5, processed for immunofluorescence and stained with antibodies to the FLAG-epitope (green), and to Pex14, a peroxisomal membrane protein (red). (**B**) Peroxisomal localisation of endogenous ACBD5 in COS-7 cells. Cells were processed for immunofluorescence using anti-ACBD5 (green) and anti-catalase (red) antibodies.

(**C, D**) The PTS1-containing protein Acyl-CoA Dehydrogenase 11 (ACAD11) localises to peroxisomes. (**C**) Peroxisomal localisation of expressed Myc-ACAD11 in COS-7 cells. Cells were transfected with Myc-ACAD11, processed for immunofluorescence and stained with antibodies to the Myc-epitope (green), and to Pex14 (red). (**D**) Peroxisomal localisation of endogenous ACAD11 in HepG2 cells. Cells were processed for immunofluorescence using anti-ACAD11 (green) and anti-Pex14 (red) antibodies. Overlays (merge) are shown on the right.